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Effect of Maturation Media on Male Pronucleus Formation in Pig Oocytes Matured In Vitro

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ABSTRACT The present study was carried out to examine the effect of maturation media on male pronucleus formation of pig oocyte matured and fertilized in vitro. Follicular oocytes collected from prepubertal gilts at a local slaughter house were cultured (36 h) in three different media (mTCM-199, Waymouth MB 752/1, and mTLP–PVA), fertilized in vitro, and assessed for nuclear maturation and male pronucleus formation. The addition of 10% (v/v) pig follicular fluid (pFF) to maturation media significantly increased the rate of nuclear maturation of pig oocytes ($P < 0.01$), whereas the rate of nuclear maturation of pig oocytes among three different media did not differ. However, the rate of male pronucleus formation of pig oocytes was significantly higher in pig oocytes matured in Waymouth MB 752/1 with or without pFF than in oocytes matured in the other two media ($P < 0.01$). In experiment 2, the addition of cysteine (the same concentration as in Waymouth medium, 0.57 mM), to mTLP–PVA significantly increased the rate of male pronucleus formation of pig oocytes compared with the control ($P < 0.01$). The results indicate that the composition of maturation medium affects the ability of pig oocytes to form male pronuclei following sperm penetration; media containing a high concentration of cysteine (possibly as a substrate of glutathione), such as Waymouth MB 752/1, can remarkably promote this ability.

Key Words: Maturation, Fertilization, Male pronucleus formation, Culture medium, Cysteine, Pig oocyte

INTRODUCTION

Fundamental studies on the cellular and molecular factors operating during oocyte maturation and fertilization provide the basis for the definition of conditions for the production of embryos in vitro and for their possible application in basic research and animal breeding.

The ability of pig oocytes to mature, fertilize, and develop in vitro has been demonstrated by several investigators (Mattioli et al., 1989; Yoshida et al., 1990; Nagai et al., 1990). However, several problems on in vitro maturation and fertilization of pig oocytes persist. One problem is the low rate of male pronucleus formation in pig oocytes matured and penetrated by sperm in vitro (Iritani et al., 1978; Nagai et al., 1984; Mattioli et al., 1988; Yoshida et al., 1990). Meanwhile, it has been reported that the co-culture of oocytes with follicular cells (Mattioli et al., 1988) and the culture of oocytes in pig follicular fluid (Naito et al., 1988) improve the ability of pig oocytes to form male pronucleus following sperm penetration in vitro. In addition, the choice of the culture medium used for maturation of mouse oocytes has a significant impact on the frequency of successful development (Eppig et al., 1990; Van de Sandt et al., 1990). The effect of culture media on nuclear maturation of pig oocytes in vitro has been reported by several investigators (Tsafriri and Channing, 1975; Sato et al., 1977; Racowsky and McGaughey, 1982; Eng et al., 1986; Yoshida et al., 1989); however, similar studies on male pronucleus formation in pig oocytes have not been reported.

The objective of this study is to examine the effect of maturation media on male pronucleus formation in pig oocytes matured and penetrated by sperm in vitro.

MATERIALS AND METHODS

The methods for in vitro maturation and fertilization of oocytes were based on those described by Yoshida et al. (1990).

Collection of Oocytes

Ovaries were collected from prepubertal gilts at a local slaughter house and transported to the laboratory in 0.9% (w/v) NaCl containing 100 mg kanamycin sulfate/L (Meiji Seika, Tokyo, Japan) at 35°C. Within 2 h of slaughter, the follicular contents were recovered by aspiration, using a 21-gauge needle (Terumo Co., Tokyo, Japan) and a 5-ml disposable syringe (Nipro, Osaka, Japan), from the follicles (2–5 mm in diameter). The oocyte–cumulus complexes were gathered from the follicular contents and were washed twice with Medium PB1 (Whittingham, 1971) and the maturation medium, respectively. Only oocytes possessing a compact cumulus mass and evenly granulated ooplasm were selected for the experiments. After removal of oocytes, pFF was pooled and centrifuged to remove granulosa cells (1,500*g*, 30 min, room temperature). Cell-free pFF was filtrated with 0.8-μm, 0.45-μm, and 0.2-μm membrane filters (Advantec Toyo Co., Tokyo, Japan), respectively, and stored at −20°C for several weeks before use.

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Preparation of Spermatozoa

The sperm-rich fractions of ejaculates were obtained from a Large White boar using the gloved-hand method. Semen samples were washed three times with 0.9% (w/v) NaCl containing 100 mg bovine serum albumin/L (Sigma Chemical Co., St Louis, MO) and 100 mg kanamycin sulfate/L. Washed spermatozoa were subsequently diluted to 5×10^7 cells/ml in the fertilization medium and used for insemination.

In Vitro Maturation and Fertilization

Oocytes (10–15) were transferred to a droplet of maturation medium (0.2 ml) under paraffin oil (Nakarai Tesque, Inc., Kyoto, Japan) in a polystyrene dish (35 mm) (Becton Dickinson Labware, Oxnard, CA) and cultured for 36 h. After incubation, oocytes (25–35) with expanded cumulus mass were transferred to fertilization medium (2.0 ml) covered with paraffin oil. The spermatozoa were introduced into the fertilization medium for a final concentration of $2.5–5 \times 10^4$ cells/ml. After 7 to 8 h incubation, oocytes were washed twice with embryo culture medium, and groups of 5–10 oocytes were transferred to droplets of embryo culture medium (0.1 ml) under paraffin oil and cultured until 18 h after insemination.

Assessment for Maturation and Pronucleus Formation

Oocytes (18 h postinsemination) were fixed with acetic alcohol (methanol and acetic acid, 3:1, v/v) and stained with 1% aceto-orcein. The stages of oocytes were determined by means of Nomarski differential interference microscopy (Olympus); oocytes with a polar body were regarded as matured, matured oocytes having both female pronucleus and the enlarged sperm head with sperm tail or both male (sperm tail), and female pronuclei were regarded as penetrated.

Culture Media and Culture Conditions

Three culture media were used for in vitro maturation of oocytes in this study: (1) modified (m) TCM-199 with Earle's salts (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 0.91 mM sodium pyruvate (Sigma), 3.05 mM glucose (Wako Pure Chemical Co., Osaka, Japan), 2.92 mM calcium lactate (Nakarai) as described by Yoshida et al. (1989; 1990); (2) Waymouth MB 725/1 (Sigma); (3) mTLP–PVA (Tyrode–lactate–pyruvate–polyvinylalcohol) supplemented with 1.0 mM glutamine (Sigma), 0.2 mM isoleucine (Sigma), 0.05 mM methionine (Sigma), and 0.1 mM phenylalanine (Sigma) as described by Bavister (1989). The pH of each media after equilibration with 5% CO_2 in air was 7.4. All media were supplemented with 10 IU pregnant mare serum gonadotropin/ml (Teikoku Zoki Co., Tokyo, Japan), 10 IU human chorionic gonadotropin/ml (Sankyo Co., Tokyo, Japan), 1 μg estradiol–17 β/ml (Sigma), 100 μg kanamycin sulfate/ml, and 10% (v/v) fetal calf serum (FCS) (GIBCO, Grand Island, NY). Unless the media indicate, the concentration of pFF added to maturation medium was 10% (v/v).

The fertilization medium consisted of 90% (v/v) mTCM-199 supplemented with 2 mM caffeine (Wako), 100 μg kanamycin sulfate/ml, and 10% (v/v) FCS. The embryo culture medium consisted of 90% (v/v) TCM-199 with Earle's salts supplemented with 0.27 mM sodium pyruvate, 21.6 mM sodium lactate (60% syrup; Sigma), 100 μg kanamycin sulfate/ml, and 10% (v/v) FCS.

In vitro maturation, fertilization, and embryo culture were performed at 38.5°C in an atmosphere of 5% CO_2 in air. The handling of oocytes and embryos under the microscope was performed at 38°C on a heated stage (Kitazato Supply Co., Tokyo, Japan).

Experiment 1. This experiment determined the effects of maturation media and pFF on in vitro maturation and fertilization of pig oocytes. Oocytes were cultured for 36 h in three maturation media with or without pFF supplementation before oocytes were transferred into the fertilization medium.

Experiment 2. This experiment was carried out to examine the effects of cysteine and glutathione added to maturation medium on in vitro maturation and fertilization of pig oocytes. Oocytes were cultured for 36 h in mTLP–PVA (10% pFF) supplemented with or without 0.57 mM L-cysteine (Sigma) and with or without 0.05 mM glutathione (r-glutamylcysteinylglycine; GSH, Sigma), before insemination.

Statistics

Experiments 1 and 2 were replicated five and three times, respectively, and data from each experiment were pooled. In each experiment, oocytes were allocated equally among the experimental groups. The statistical significance of the results was evaluated by the χ^2 test with the Yates correction for continuity.

RESULTS

Experiment 1

As shown in Table 1, the rate of male pronucleus formation was significantly higher ($P < 0.01$) in oocytes matured in Waymouth medium than in those in matured in mTCM-199 or mTLP–PVA. Also, the rate of male pronucleus formation was higher in oocytes matured in mTCM-199 than in those matured in mTLP–PVA. When pFF was added to the maturation media, the rate of and nuclear maturation of oocytes were significantly higher ($P < 0.01$) than the rate for oocytes in media without pFF. By contrast, pFF had no effect on the rate of male pronucleus formation. The efficacy of pFF was similar among the three maturation media.

Experiment 2

The effects of cysteine and glutathione (GSH) addition to maturation medium on nuclear maturation, penetration, and pronucleus formation in pig oocytes are presented in Table 2. The rates of nuclear matura-

TABLE 1. Effects of Maturation Media and Pig Follicular Fluid (pFF) on Nuclear Maturation, Penetration, and Pronucleus Formation in Pig Oocytes

Media	Addition of pFF	No. (%) of oocytes		
		Nuclear maturation	Sperm penetration[a]	Male pronucleus formation[b]
mTCM-199	−	91/134 (68)*A	80/91 (88)	44/80 (55)*A
	+	120/130 (92)*B	104/120 (87)	65/104 (63)*A,C
Waymouth	−	86/134 (64)*A	81/86 (94)	70/81 (86)*B
	+	117/124 (94)*B	110/117 (94)	100/110 (91)*B
mTLP-PVA	−	106/152 (70)*A	92/106 (87)	41/92 (45)*A,D
	+	104/118 (88)*B	98/104 (94)	46/98 (47)*A,D

[a]In matured oocytes.
[b]In matured and penetrated oocytes.
*$P < 0.01$ for A vs. B within each column; $P < 0.05$ for C vs. D within each column.

TABLE 2. Effects of Cysteine and Glutathione (GSH) Addition to mTLP-PVA on In Vitro Maturation, Penetration and Pronucleus Formation in Pig Oocytes

Addition		No. (%) of oocytes		
Cysteine	GSH	Nuclear maturation	Sperm penetration[a]	Male pronucleus formation[b]
−	−	65/77 (84)	64/65 (99)	13/64 (20)*A
+	−	64/75 (85)	63/64 (98)	59/63 (94)*B,C
−	+	67/79 (85)	66/67 (99)	39/66 (59)*B,D
+	+	62/79 (89)	58/62 (94)	54/58 (93)*B,C

[a]Sperm penetration.
[b]In matured and penetrated oocytes.
*$P < 0.01$ for A vs. B and for C vs. D within each column.

tion and sperm penetration of oocytes were high in all media, whereas the rates of male pronucleus formation were significantly higher in oocytes matured in the media with supplemental cysteine and/or GSH than in those matured in the control medium ($P < 0.01$). Moreover, the rates of male pronucleus formation were significantly higher in oocytes matured in media containing cysteine with or without GSH than in those matured in the medium with GSH alone ($P < 0.01$).

DISCUSSION

The results of this study show that the composition of media for in vitro maturation of pig oocytes affects the ability of oocytes to form the male pronucleus after sperm penetration. Both Waymouth medium and mTLP–PVA with cysteine (0.57 mM) were effective in promoting this ability. The limited capacity of male pronucleus formation in pig oocytes matured and penetrated by sperm in vitro has been described by several investigators (Iritani et al., 1978; Nagai et al., 1984; Mattioli et al., 1988; Yoshida et al., 1990). The rates of male pronucleus formation of pig oocytes matured in Waymouth medium or in mTLP–PVA with cysteine were considerably higher than those reported earlier.

The results of experiment 1 are consistent with the previous reports that Waymouth medium had a beneficial effect on in vitro maturation of mouse oocytes (Eppig et al., 1990; Van de Sandt et al., 1990). These investigators found that in comparison with seven other culture media, Waymouth medium was effective in accelerating the time of polar body emission and resulted in the highest frequency of fertilized mouse ova subsequent to the blastocyst stage. In the present study with pig oocytes, we did not detect any difference in the percentage of oocytes that emitted polar bodies after in vitro maturation, but the time of polar body emission was not evaluated.

Waymouth medium was originally developed as a defined synthetic medium for the cultivation of mouse L929 cells (Waymouth, 1959); it differs both qualitatively and quantitatively from mTCM-199 in numerous components. Of particular interest is the higher concentration of GSH and cysteine in Waymouth medium than in mTCM-199. GSH is major intracellular free thiol. It has important biological functions during cellular proliferation, amino acid transport, and synthesis of protein and DNA, and it protects cells against oxidation (Meister and Anderson, 1983). The synthesis of GSH during oocyte maturation is reported to be a prerequisite for sperm chromatin decondensation, hence for male pronucleus formation, after sperm penetration of mouse (Calvin et al., 1986) and hamster oocytes (Perreault et al., 1988; Perreault, 1990). In addition, pig oocytes matured in Waymouth medium that contained the GSH synthesis inhibitor, buthionine sulfoximine (Griffith and Meister, 1979), exhibited a reduced ability to form male pronuclei after sperm penetration (unpublished observation). Therefore, it appears likely

that Waymouth medium is effective for synthesis of sufficient GSH during in vitro maturation of pig oocytes for sperm decondensation and male pronucleus formation in vitro. Investigations are in progress to determine the GSH concentration in pig oocytes during maturation.

In experiment 2, the addition of cysteine to mTLP–PVA, to provide the same concentration as in Waymouth medium, was more effective for promoting male pronucleus formation in pig oocytes than was the addition of GSH. This finding is consistent with the report of De Felici et al. (1987) that permeability of plasma membranes to GSH is low and the findings of Meister (1983) that intracellular concentrations of GSH were dependent on the availability of cysteine.

In the current study, the addition of pFF to maturation media was effective for enhancing the rate of nuclear maturation; however, pFF was not effective for promoting male pronucleus formation. While these results are consistent with our previous observations (Yoshida et al., 1990, 1991), they conflict with the report of Naito et al. (1988), which indicated that pFF enhanced male pronucleus formation. These conflicting results might be due to differences in culture conditions or to interactions of pFF with specific components that make up the media used in the experiments.

In conclusion, these results indicate that the addition of cysteine to the maturation medium is an important factor for enhancing the ability of pig oocytes to form male pronuclei after sperm penetration. Whether cysteine is acting by permitting GSH synthesis by the oocyte, as we have hypothesized, deserves additional investigation.

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